International Journal of Medicine and Pharmaceutical Sciences (IJMPS)
ISSN(P): 2250-0049; ISSN(E): 2321-0095
Vol. 5, Issue 2, Apr 2015, 15-22

DEPLETION OF ALBUMIN FROM HUMAN SERUM FOR HIGH THROUGH PUT PROTEOMICS STUDIES

KAUSAR SABOOHI[1], ASIFA ALIA[2], SANA RAZA[3], SAMINA N SHAKEEL[4] & BUSHRA CHAUDHRY[5]

[1,2,3,5]Department of Biological and Biomedical Sciences. The Aga Khan University Medical College, Stadium Road, Karachi, Pakistan

[3,4]Department of Biochemistry, Quaid-e-Azam University, Islamabad, Pakistan

ABSTRACT

Sample preparation from serum and plasma to use in proteomics, mass spectrometry (MS) and two dimensional gel (2D gel) based analytical methods, has received a greater focus to get reliable and reproducible data. Particular emphasis has been placed on the removal of the most abundant proteins such as albumin. Thecommercial kits based on affinity chromatography are expensive for a large sample size and have shelf life limitations. Our objective through this study was to develop an efficient and cost effective method for removal of albumins. Blue-sepharose, a 6% cross-linked agarose with a particle size range of 45-165µm, was prepared in a syringe column. Total proteins of the serum were allowed to bind to the column and five different elution buffers were used to elute proteins devoid of albumin/ immunoglobulin. The eluted proteins were subjected to gel fractionation. Our results indicated that binding capacity of the sepharose slurry to serum albumin was approximately 4mg/ml. Moreover, buffer "C" (0.1M Tris, 0.04M BME pH 7.5) showed the best retention of albumin on the column as indicated by the quality of albumin free eluted proteins from diabetic patients and healthy control group. Further, we observed that a careful selection of the elution buffer was required for a particular set of proteins to be removed from serum.A simple and cost effective micro-column, albumin purification method from serum is described. This provided good quality and quantity of albumin depleted samples for analysis of serum proteome complexity.

KEYWORDS: Diabetic Patients, Serum, Blue-Sepharose, Albumin Removal, Proteomic Study

INTRODUCTION

The presence of albumin and immunoglobulin's in body fluids such as serum & plasma make a differential analysis of proteins very difficult in proteomics study and nearly impossible in 2-D gel electrophoresis. In blood serum samples these proteins are abundant and make up between 30-50 % of the total protein contents (Bradford 1976). Albumin may not effect some of the high abundant proteins present in the serum which contribute <0.1 % of total number of proteins. But, Albumin and globulins may mask the low abundant proteins of serum such as osteopotin, prostate-specific antigen, various interleukins and cytokines which are the biomarkers for different diseases. These are also impending biomarker for malignant and non-malignant diseases(Huang et al. 2005). These proteins are found in micro to Pico molar concentration(Huang et al. 2005; Seam et al. 2007) and because of their small concentrations, these are difficult to spot on SDS-PAGE and 2D gel electrophoresis.

Albumin and globulin mask many interesting proteins in their neighborhood as well because of their high concentration, wide isoelectric point and molecular weight ranges. In addition, their degradation products can be found anywhere in the 2-D gels. Since there is limit on the total amount of protein which can be applied to the immobilized pH gradient, it is clear that many low abundant proteins cannot be detected. To overcome this difficulty, one must use enough amounts of proteins of interest after efficient removal of albumin / globulin proteins. Therefore, pre-fractionation of biological samples especially for serum and plasma becomes necessary under certain situations of protein analysis.

There are several commercial kits available to overcome this problem such as Enchant TM Albumin Depletion Kit from VWR International (vwr.com), Montage Albumin Deplete Kit from Millipore (Siegmund et al. 2009), Spin IgY-12 kit from GenWay (Seam et al. 2007), HPLC Hu-PL7 from Agilent , human serum albumin [immuno-affinity column (IAC)] (Magagnotti et al. 2010),Q proteome Albumin/ IgG Depletion Kit from Qiagen, ProteoPrep Immuno-affinity Albumin and IgG Depletion Kit from Sigma. Many of these kits are composed of high-binding capacity antibody based resin (Magagnotti et al. 2010). These kits are mostly expensive. The cost ranged from $10-20 per sample. To use commercially available kits to achieve the objectives is fine for well-funded research groups. However our procedure is equally well for use in case of large number of samples if required.

Our method is economical for the partial removal of albumin form the serum, using Sepharose CL-6 (Welch et al. 1989; Kawano 1994), a protein binding resin which has properties of affinity and ion exchange binding, costing several times lower than the commercially available counterparts. Sepharose CL-6B use is a method of choice by many researchers to purify many proteins especially NAD/ NADH requiring enzymes during protein purification study, independent of total proteome analysis (Welch et al. 1989). We have also used Sepharose CL-6B to remove serum albumin from diabetic patients and healthy control (without diabetes) samples individually, required to analyze on SDS-PAGE and 2D gel electrophoresis. It was used for analytical purification in our study, for small amount of protein from both kind of samples (diabetic patients and healthy controls). The cross linked sepharose followed the principle of covalent chromatography and protein was eluted by the addition of reducing agent in the buffer.

MATERIALS AND METHODS

Serum Samples

The protocol was approved by the ethics committee of The Aga Khan University; Karachi, Pakistan. The human blood sample was collected in gel collection tubes by healthy controls (non-diabetics) and patients (diabetics in age group of >35 years, with duration of diabetes 5-10 years), who provided written informed consent. Serum was separated from blood cells after centrifugation of samples at 2000 rpm for 10 min.

Column Preparation: Packing of Blue Sepharose CL-6B

Blue- Sepharose powder (1g) was hydrated with distilled water (50ml) for overnight at 4^0C. The slurry was resuspendedin binding buffer (50ml, 0.1M Tris pH 7.5, 0.5M NaCl, 1mM EDTA**)** for 12hr. Columns were prepared using insulin syringe. The end of the syringe was cut and glass wool was inserted to plug the bore. Blue Sepharose, saturated with binding buffer was poured in the syringe to 1 ml. it was allowed to stand in 15 ml falcon tube for 10 min. Excessive binding buffer was allowed to flow through manually. Additional slurry was poured to fill the column. The procedure was repeated till it was filled up to 0.5CC mark. Column was packed to 3 cm of length and was ready to use.

Binding of Protein on Mini Column

Total 2mg of serum protein was taken in 3ml volume in binding buffer. These serum proteins in binding buffer (0.66mg/ml) were then subjected to sonication for 15 sec for three times on ice. Column was set on 15ml falcon tube and sonicated serum (600ul) was loaded on it. Total five columns were loaded with 2mg of protein for each in a volume of 600ul, to test five different elution buffers. Columns were reloaded twice with the filtrate by adding back on the columns. Columns were transferred to the new 15ml falcon tubes and were marked as A-E, for identification of the elution buffers.

Elution of Albumin Free Proteins

The buffers A-E had the following compositions: Elution Buffer A (25 mM DTT, 100mM Tris pH7.5), Elution Buffer B (20 mM BME, 100mM Tris pH7.5), Elution Buffer C (40 mM BME, 100mM Tris pH7.5), Elution Buffer D (25 mM L-Cysteine, 100mM Tris pH7.5) and Elution Buffer E (50 mM Glutathione, 100mM Tris pH7.5).Serum proteins were eluted in these different buffers and proteins were estimated using Bradford's method (Bradford 1976). Protein binding and elution method is shown in figure 1

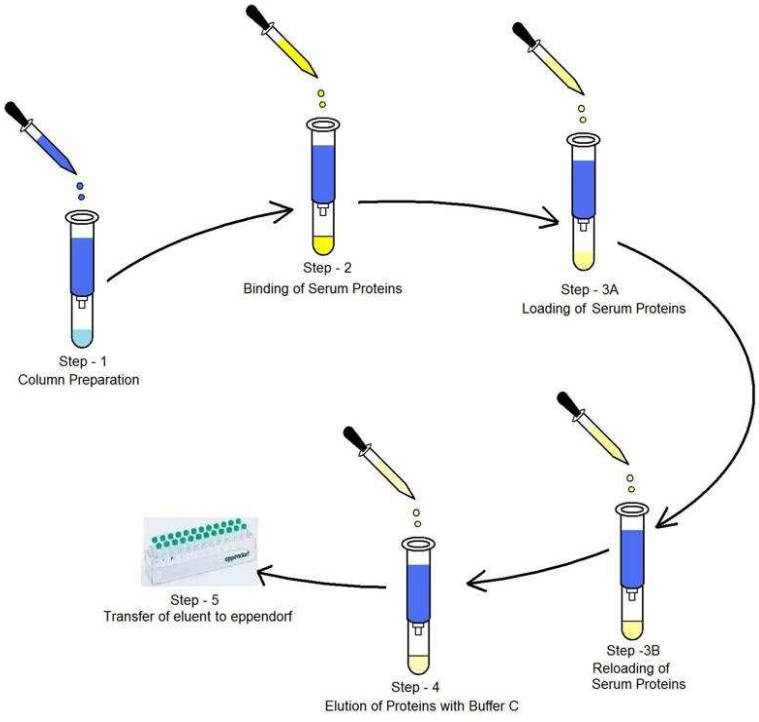

Figure 1: Schematic Diagram Indicating Steps Involved in Removal of Albumin from Serum

Protein Analysis

Albumin free proteins (60 ug) were lyophilized and dissolved in 0.1M Tris pH 7.5 (20ul) and fractionated through 10% SDS-PAGE(Laemmli 1970). Proteins were stained with coomassie brilliant blue R-250 (Neuhoff et al. 1988).

RESULTS & DISCUSSIONS

We have run serum samples without depletion of the albumins /globulins in 10% SDS-PAGE which did not show enough number of the fractionated proteins as clear from the figure (Figure 2).Majority of the proteins wereeither trapped or bound with albumins orglobulins andcould hardly move through the cross links of the polyacrylamide gel.The excess

amount of the globulins and albumins masked the detectable limits of the lower quantities of other proteins. The big blob of proteins in the upper portion of the gel clearly indicatedthe presence of excess amount of the albumin and globulin (Figure 2).

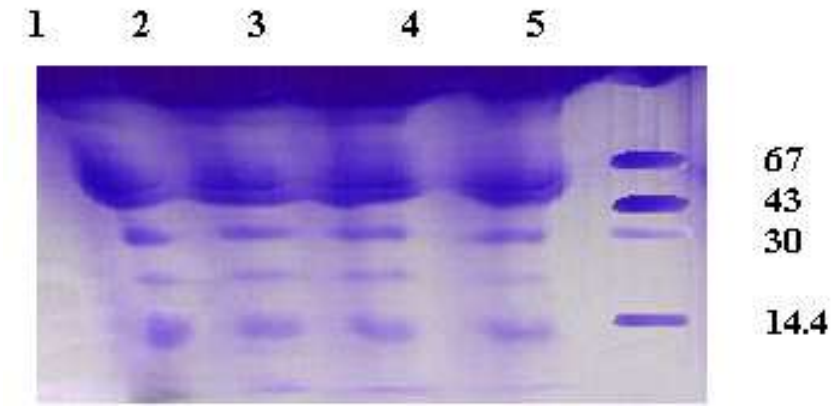

Figure 2: SDS-PAGE (10%) Showing Protein Fractionation of Different Serum Samples Stained with Coomassie. Lane 1- 4, Total Serum Proteins without Removal of Albumin and Lane 5, Molecular Weight Marker

To overcome this problem we prepared the Sepharose CL-6B mini column as described above (Figure 1).

We have used Sepharose CL-6B (Amersham Pharmacia) which is a beaded form of agarose-based gel filtration matrix and is cross linked, has the better quality of flow characteristics and is resistant to organic solvents. It has a broad fractionation range and is suitable for characterizing or cleaning up samples containing components of diverse molecular weights. Blue Sepharose has been recommended for use in protein purifications from long times (Travis et al. 1976; Angal and Dean 1977; Alam et al. 2009). It has been recommendedusing in 30 cm long columns, in preparative chromatography, for removal of albumins from the large samples during protein purification(Laemmli 1970; Neuhoff et al. 1988). Cibachron blue is a dye attached to Sepharose through a 6% cross-linked agarose with a particle size range of 45-165µm. Cibachron blue binds with albumin, interferon, lipoproteins and blood coagulation factors and several enzymes requiring NAD+ as cofactors (Kawano 1994), whereas, other proteins can successfully eluted in different elution buffers.

We have used the same resin and developed the method at micro levels preparing maximum of 3cm long column. It had followed the principle of covalent chromatography by which covalent bonds are formed between the gel and molecules in the mobile phase. Thiopropyl Sepharose 6B reacts with solutes containing thiol groups to form a mixed disulphide and release 2-thioridone, the solute then covalently linked to the gel from which it can be subsequently eluted by addition of a reducing agent e.g. β-mercaptoethanol (BME), dithiothretol (DTT), or glutathione. To minimize the risk of disulphide formation between molecules in solution additional oxidizing agents are generally not used. We allowed the serum proteins to attach with the resins and then eluted the albumin free proteins.

Our results showed depletion of albumin from serum. We estimate that 90% of albumin was successfully removed from the samples. We have used five different buffers designated as Buffers A, B, C, D and E.Albumin has been depleted from serum proteins with all buffers but buffer C showed the best results when quantified and fractionated in SDS-PAGE (Figure 3). Buffer D which contained25mM L-cysteine remained unable to elute any protein from serum. The sample eluted with buffer “C” showed high amount of protein and fractionated sharplyon SDS-Page gelas seen in gel stained with coomassie (Figure 3)

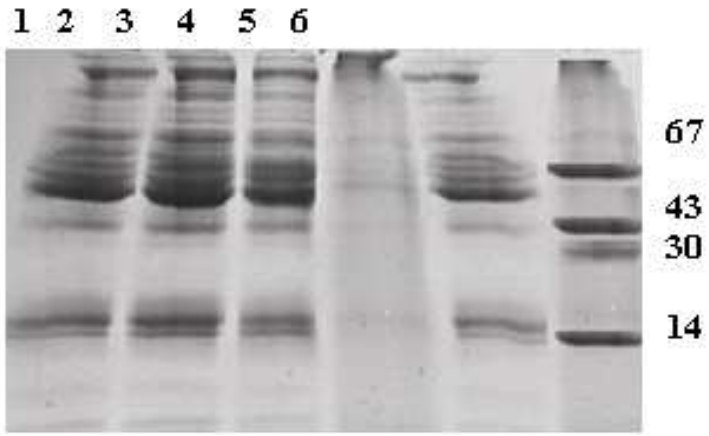

Figure 3: Protein Fractionation of a Serum Sample Eluted from Different Elution Buffers.

Lane 1, Elution buffer A, Lane 2, Elution buffer B, Lane 3, Elution buffer C, Lane 4, Elution Buffer D, Lane 5, Elution Buffer E and Lane 6, Molecular weight marker.

The appearance of protein bands in sample eluted with equal volumes of buffers A, B and E were not clear when compared to samples eluted with buffer C. Buffer C was composed of 40mM β-mercaptoethanol and 100mMTris wasconsidered as the most efficient elution buffer. All eluted proteins were detected with Bradford method of protein detection. Buffer "C" was then selected and used for removal of albumin from samples of diabetic population (Figure 4).

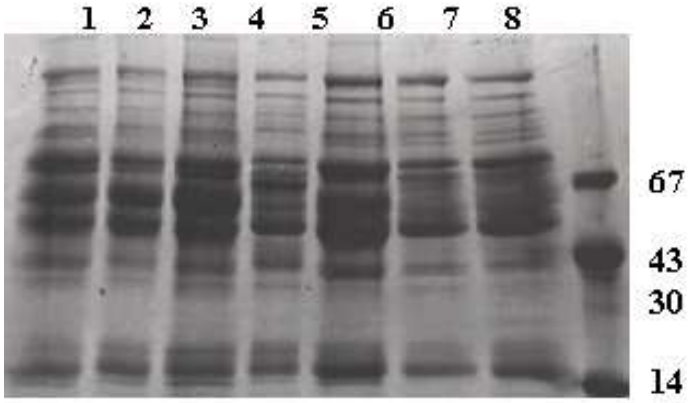

Figure 4: SDS-PAGE (10%) Showing Protein Fractionation of Different Serum Samples Stained with Coomassie after Removal of Albumin through Blue Sepharose Minicolumns Using Buffer C. Lane 1- 7, Diabetic Patient's Albumin Free Serum Samples and Lane 8, Molecular Weight Marker

Results indicated Sample eluted with buffer A, B and E showed good results when fractionated through SDS-PAGE. Our elution buffer A contained 25mM DTT, elution buffer B contained 20mM β-mercaptoethanol, and elution buffer E contained 50mM Glutathione. Elution buffer D containing 25mM L-Cysteine showed not a single protein band in protein gels which means that it failed to elute any protein, probably the exposed disulphide bridges of proteins were strongly bound to albumins and globulins and buried bound were forming inter-chain linkages with globulin. The L-cystein was unable to dissociate the protein polymers and disruption of quaternary structures for release from the column. There is also possibility that L-cysteine gets oxidized quickly. Buffer C containing 40mM β-mercaptoethanol showed the best results. In our study 40mM BME is an optimal concentration at which all other proteins were detached

from albumin through mini column. All above buffers (A-E) were of the same pH 7.5 and concentration of Tris(100mM) but with different reducing agents. These homemade mini columns proved to be very efficient in removing 80-90 % albumins from human serum according to our estimate.

CONCLUSIONS

It is concluded that albumin from biological fluids can be removed efficiently by blue-Sepharose mini columns for depletion of albumin from serum. This can be achieved using buffers with varying concentration of different reducing agents. Elution of albumin free serum proteins is most efficient in buffers containing BME. In our opinion this is an economical way for high through put study and in biomarker detection

ACKNOWLEDGEMENTS

We thanks for financial support of consumables from the Department of Biological & Biomedical Sciences, The Aga Khan University.

REFERENCES

1. Alam, M., Mahajan, M., Raziuddin,M.,Singh,T. P.,Yadav,S. (2009). Proteomics-based approach for identification and purification of human phosphate binding apolipoprotein from amniotic fluid. Genet Mol Res. 8(3), 929-937.
2. Angal, S., Dean, P. D.(1977). The effect of matrix on the binding of albumin to immobilized Cibacron Blue. Biochem, J. 167(1), 301-303.
3. Bradford M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal, Biochem. 72, 248-254.
4. Huang, L., Harvie, G., Feitelson, J. S., Gramatikoff, K., Herold, D.A et al. (2005). Immunoaffinity separation of plasma proteins by IgY microbeads: meeting the needs of proteomic sample preparation and analysis. Proteomics. 5(13), 3314-3328.
5. Kawano, T., Kozutsumi, Y., Kawasaki, T., Suzuki, A. (1994). Biosynthesis of N-glycolyneuraminic acid containing glycoconjugates. Purification and characterization of the key enzyme of the cytidine monophospho-N-acetylneuraminic acid hydroxylation system. J,BiolChem. 269, 9024.
6. Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227(5259), 680-685.
7. Magagnotti, C., Fermo, I., Carletti, R. M., Ferrari, M., Bachi, A. (2010). Comparison of different depletion strategies for improving resolution of the human urine proteome. Clin Chem Lab Med. 48(4), 531-535.
8. Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue Gâ€250 and Râ€250. Electrophoresis. 9(6), 255-262.
9. Seam, N., Gonzales, D.A., Kern, S.J., Hortin, G.L., Hoehn, G.T. et al. (2007). Quality control of serum albumin depletion for proteomic analysis. Clin, Chem. 53(11), 1915-1920.
10. Siegmund, R., Kiehntopf, M., Deufel, T. (2009). Evaluation of two different albumin depletion strategies for

improved analysis of human CSF by SELDI-TOF-MS. Clin, Biochem. 42(10-11), 1136-1143.

11. Travis, J. Bowen, J., Tewksbury, D., Johnson, D., Pannell, R. (1976). Isolation of albumin from whole human plasma and fractionation of albumin-depleted plasma. Biochem, J. 157(2), 301-306.
12. VWR.com VI, 911 Commerce Ct. Baffalo Grove, IL 60089, 847-520-6170.
13. Welch, R.W., Rudolph, F. B., Papoutsakis, E. T. (1989). Purification and characterization of the NADH-dependent butanol dehydrogenase from Clostridium acetobutylicum (ATCC 824). Archives of biochem, and biophysics. 273(2), 309-318.